

## Cytochrome P4501A induction in avian hepatocyte cultures exposed to polychlorinated biphenyls: Comparisons with AHR1-mediated reporter gene activity and *in ovo* toxicity

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### ABSTRACT

Avian-specific toxic equivalency factors (TEFs) were developed by the World Health Organization to simplify environmental risk assessments of dioxin-like compounds (DLCs), but TEFs do not account for differences in the toxic and biochemical potencies of DLCs among species of birds. Such variability may be due to differences in species sensitivity to individual DLCs. The sensitivity of avian species to DLCs was recently associated with the identity of amino acids 324 and 380 in the aryl hydrocarbon receptor 1 (AHR1) ligand binding domain. A luciferase reporter gene (LRG) assay, measuring AHR1-mediated induction of a cytochrome P450 1A5 (CYP1A5) reporter gene, in combination with a species' AHR1 ligand binding domain sequence, were also shown to predict avian species sensitivity to polychlorinated biphenyls (PCBs) and PCB relative potency in a given species. The goals of the present study were to (1) characterize the concentration-dependent effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and PCBs 126, 77, 105 and 118 on induction of ethoxyresorufin *O*-deethylase (EROD) activity and CYP1A4/5 mRNA in chicken, ring-necked pheasant and Japanese quail embryo hepatocytes and (2) compare these *in vitro* results to those previously generated by the LRG assay and *in ovo* toxicity studies. EROD activity and CYP1A4/5 mRNA expression data support and complement the findings of the LRG assay. CYP1A enzyme activity and mRNA expression were significantly correlated both with luciferase activity and *in ovo* toxicity induced by PCBs. Relative potency values were generally similar between the LRG and EROD assays and indicate that the relative potency of some PCBs may differ among species.

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### Introduction

Dioxin-like compounds (DLCs), which include polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans and certain polychlorinated biphenyl (PCB) congeners, are a group of structurally-related chemicals that cause toxicity subsequent to binding and activation of the aryl hydrocarbon receptor (AHR) (Denison et al., 2011; Okey, 2007). Because polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans and PCBs exist as complex mixtures of congeners within environmental and biological samples, the toxic equivalent (TEQ) approach was developed by the World Health Organization (WHO) to

simplify risk assessment of DLC mixtures (van den Berg et al., 1998). Under this framework, toxic equivalency factors (TEFs) are used to represent the toxic potency of a DLC relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Relative potency (ReP) values from the literature were evaluated by an expert panel to derive TEFs specific to mammals, fish and birds for each DLC (van den Berg et al., 1998); however, these TEFs do not consider differences in DLC potency among species within these classes of animals. For example, 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) is equipotent to TCDD in the chicken (*Gallus gallus domesticus*) but it is up to 6 and 30 times more potent than TCDD in the ring-necked pheasant (*Phasianus colchicus*) and Japanese quail (*Coturnix japonica*), respectively (Cohen-Barnhouse et al., 2011; Farmahin et al., 2012; Hervé et al., 2010).

Differences in the relative potency of PeCDF observed among species are likely related to differences in species sensitivity to individual DLCs, as Japanese quail embryos were 45 times less sensitive to TCDD but only 7 times less sensitive to PeCDF when compared to chicken embryos (Cohen-Barnhouse et al., 2011). Avian species sensitivity to DLCs has been associated with the identity of amino acids at sites 324 and 380 within the AHR1 ligand binding domain (Farmahin et al., 2012;

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Karchner et al., 2006), and the results of AHR1 ligand binding domain sequencing of 86 species of birds indicates that species can be divided into three main groups based on their AHR1 genotype: type 1 (Ile324\_Ser380), type 2 (Ile324\_Ala380) and type 3 (Val324\_Ala380) (Head et al., 2008; Farmahin et al., in press).

*In vitro* measures of AHR1 activation, such as cytochrome P4501A (CYP1A) induction, are good predictors of overt DLC toxicity (Head and Kennedy, 2010). Relative to egg injection studies, the use of primary avian hepatocyte cultures for measuring CYP1A enzyme activity or mRNA expression significantly reduces the number of animals required for testing the effects of DLCs. A luciferase reporter gene (LRG) assay, which measures AHR1-mediated induction of a CYP1A5 reporter gene, in combination with the knowledge of a species' AHR1 genotype has recently been shown to predict DLC toxicity in potentially any avian species and requires the lethal sampling of even fewer individuals than *in vitro* hepatocyte screening studies (Manning et al., 2012; Farmahin et al., in press).

The goals of the present study were (1) to characterize the concentration-dependent effects of TCDD and PCBs 126, 77, 105 and 118 on induction of ethoxyresorufin O-deethylase (EROD) activity and CYP1A4/5 mRNA expression in primary hepatocyte cultures from three domestic species representative of each major AHR1 type, namely chicken (type 1), ring-necked pheasant (type 2) and Japanese quail (type 3), and (2) to calculate relative species sensitivity and relative PCB potency among the three species for comparison with results from the LRG assay (Manning et al., 2012) and *in ovo* toxicity studies from the literature. In addition, it was previously suggested that contamination of *mono-ortho* PCB solutions with more potent AHR agonists results in overestimation of their ReP values (Peters et al., 2006; van den Berg et al., 2006). Therefore, the effects of reagent-grade and purified solutions of PCB 105 and 118 on EROD activity and CYP1A4/5 mRNA expression were also compared.

## Materials and methods

### Purification of mono-ortho PCBs

A complete description of the purification of PCB 105 and 118 is provided elsewhere (Manning et al., 2012). In brief, purified crystalline, reagent-grade samples of PCB 105 and 118, hereafter referred to as PCB 105-RG and 118-RG, were obtained by Wellington Laboratories (Guelph, ON, Canada) and analyzed by high resolution gas chromatography/low resolution mass spectrometry (HRGC/LRMS) to confirm their identity and purity. PCB 105-RG and 118-RG were purified by thin layer chromatography and carbon column chromatography to obtain purified crystalline samples of PCB 105 and 118, hereafter referred to as PCB 105-P and PCB 118-P.

### Preparation and analysis of TCDD and PCB solutions

**Chicken and Japanese quail studies.** Serial dilutions of PCB 105-P, 118-P (Wellington Laboratories; >99% pure; lot number 060611), 105-RG, 118-RG and 126 (Wellington Laboratories; >99% pure; lot number 031711) were prepared from dimethyl sulfoxide (DMSO) stock solutions with a nominal concentration of 196 µg/ml. Actual concentrations were 202.8, 207.2, 196.9, 198.9 and 205.3 µg/ml for PCB 105-P, 105-RG, 118-P, 118-RG and 126, respectively. Identification and quantification of PCBs were performed by HRGC/LRMS as described in Manning et al. (2012). PCB 105-P, 118-P, 105-RG and 118-RG solutions were spiked with <sup>13</sup>C-labelled PCDD, PCDF and PCB surrogates and analyzed by high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS) by use of isotopic dilution in order to test for the presence of PCDDs, PCDFs and coplanar PCBs. Where present, the PCDDs, PCDFs and PCBs were quantitated by internal standard methods.

A detailed description of the preparation and analysis of TCDD stock solutions is provided elsewhere (Hervé et al., 2010). Serial dilutions of

TCDD were prepared from a DMSO stock solution with a nominal concentration of 80 µg/ml. Identification and quantification of TCDD in the stock solution were determined by isotope dilution following EPA Method 1613 by use of HRGC/HRMS. The measured concentration of TCDD was 72.9 µg/ml. Serial dilutions of PCB 77 were prepared from a stock solution with a nominal concentration of 121 µg/ml. The PCB 77 stock solution was prepared by dissolving 0.48 mg of PCB 77 (ULTRA Scientific, Kingstown, RI, USA; >99% pure; lot number A-0094) into 4 ml of DMSO.

**Ring-necked pheasant study.** Serial dilutions were prepared from TCDD, PCB 126, PCB 77, PCB 105-RG and PCB 118-RG stock solutions. The TCDD and PCB 77 stock solutions used in the chicken and Japanese quail studies were also used in the ring-necked pheasant study. PCB 105-RG (AccuStandard, New Haven, CT, USA; lot # 101905AG-AC; purity stated to be 100%), PCB 118-RG (AccuStandard; lot # 032494; purity stated to be 100%) and PCB 126 (AccuStandard, lot #061204MS-AC; 99.7% pure) were weighed (0.5–1.0 mg) and stock solutions were prepared in DMSO. The nominal concentrations of the stock solutions were 83, 160 and 153 µg/ml for PCB 105-RG, 118-RG and 126, respectively.

### Preparation and dosing of cultured hepatocytes

Primary cultures of hepatocytes were prepared from avian embryos using methods described elsewhere (Kennedy et al., 1995) and included subsequent modifications (Head and Kennedy, 2007b; Kennedy et al., 2003). All procedures were conducted according to protocols approved by the Animal Care Committee at the National Wildlife Research Centre. Reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Fertilized, unincubated chicken, ring-necked pheasant and Japanese quail eggs were obtained from the Canadian Food Inspection Agency (Ottawa, Ontario, Canada), Couvoir Simetin (Mirabel, QC, Canada) and Crazy Quails (Oshawa, Ontario, Canada), respectively. Eggs were incubated at 37.5 °C and 60% relative humidity until 1–3 days pre-hatch (19, 23 and 16 days for chicken, ring-necked pheasant and Japanese quail, respectively). Eggs were candled periodically and infertile eggs or eggs containing dead embryos were discarded. In brief, 61 chicken, 51 ring-necked pheasant and 125 Japanese quail embryos were euthanized by decapitation, and livers were removed, pooled and digested with collagenase (Head et al., 2006). Percoll (Amersham Bioscience, Uppsala, Sweden) density gradient centrifugation was used to separate erythrocytes from hepatocytes, and DNase (Roche, Laval, Quebec, Canada) treatment was carried out to prevent cell clumping. Cells were plated in 48-well culture plates by adding 25 µl of the cell suspension to 500 µl of cell culture medium 199, supplemented with insulin (1 µg/ml) and thyroxine (1 µg/ml), and incubated for 24 h at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. After 24 h, cells were treated in triplicate with in-well concentrations of TCDD ranging from 0.0003 to 10 nM, and concentrations of PCB 126, 77, 105 and 118 ranging from 0.03 to 3000 nM. After another 24 h incubation period, plates to be used for real-time quantitative PCR (QPCR) analysis were flash-frozen in powdered dry ice and stored at –80 °C until the time of analysis. Plates used for EROD assays were rinsed with approximately 200 µl/well of phosphate-buffered saline-ethylenediaminetetraacetic acid (PBS-EDTA) prior to being flash-frozen.

### Cell viability

Cell viability was determined using the Calcein-AM assay as per the manufacturer's instructions (Invitrogen-Molecular Probes, Eugene, OR, USA). Vehicle (DMSO)-treated cells were included as a positive control and 99% ethanol-killed cells were used as a negative control. A working solution was prepared by adding 3 µl of Calcein-AM to 10 mL of PBS-EDTA. The culture medium was removed and 200 µl of the Calcein-AM solution was added to each well. Plates were incubated in the dark for 45 min at room temperature and fluorescence was then

measured using a Cytofluor 2350 (Millipore, Bedford, MA, USA) fluorescence plate reader with a 485 nm excitation wavelength and a 530 nm emission wavelength. Mean cell viability was assessed after 24 h exposure for the range of concentrations administered for each compound.

#### Ethoxyresorufin O-deethylase (EROD) assays

A detailed description of the EROD assay is provided elsewhere (Head and Kennedy, 2007b; Kennedy et al., 1995). In brief, 3 replicate plates of hepatocytes per chemical were incubated at 37.5 °C in the presence of nicotinamide adenine dinucleotide phosphate (NADPH, reduced) and 7-ethoxyresorufin for 7 min. Reactions were stopped by the addition of cold acetonitrile containing fluorecamine. Standard curves of resorufin and protein were prepared on each 48-well plate for each run. Plates were analyzed for both EROD activity (excitation wavelength: 530 nm, emission wavelength: 590 nm) and total protein concentration (excitation wavelength: 400 nm, emission wavelength 460 nm) using a Cytofluor 2350 fluorescence plate reader.

#### RNA isolation and complementary DNA synthesis

Total RNA was extracted from 48-well plates using RNeasy 96 kits (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's instructions with modifications described elsewhere (Head and Kennedy, 2007a). An on-column DNase treatment was performed and, to ensure the maximal removal of genomic DNA, total RNA was treated a second time with DNase from the Ambion TURBO DNA-free kit (Ambion, Austin, TX) according to the manufacturer's instructions. Total RNA was reverse transcribed to complementary DNA (cDNA) with Superscript II Reverse Transcriptase and random hexamers (Invitrogen, Burlington, Ontario, Canada) as per manufacturer's instructions. From each plate, a control without reverse transcriptase enzyme was included to verify the absence of genomic DNA in the RNA template.

#### Quantitative Polymerase Chain Reaction (QPCR)

Multiplex QPCR assays using dual-labelled fluorescent hydrolysis probes (Hervé et al., 2010) were used to quantify CYP1A4, CYP1A5 and  $\beta$ -actin (normalizer gene) mRNA abundance in chicken and Japanese quail embryo hepatocytes exposed to TCDD and PCBs. With the exception of primers and probes, all QPCR reactions were carried out using reagents from Stratagene and either the Stratagene Mx3000P or Mx3005 instrument (Stratagene, La Jolla, CA, USA). All primers (Invitrogen) and probes (Biosearch, Novato, CA, USA) were designed and validated as previously described (Hervé et al., 2010). Taqman reactions were performed using Brilliant Multiplex QPCR Mastermix kits (Agilent Technologies, Santa Clara, CA, USA). Each 25  $\mu$ l reaction contained forward and reverse primers and probes for the genes of interest (CYP1A4, CYP1A5) and the normalizer gene ( $\beta$ -actin), 1  $\times$  Multiplex QPCR master mix, 30 nM reference dye (ROX) and 5  $\mu$ l of cDNA. The thermal profile for all reactions was as follows: 10 min at 95 °C, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min. Data were collected at the end of the 60 °C phase. Each assay was performed once with samples in duplicate. Fold changes of CYP1A4/5 mRNA expression in TCDD- and PCB-treated hepatocytes were quantified using the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001).

#### EROD and CYP1A4/5 mRNA data analysis

Fluorescence data were imported into GraphPad (GraphPad Prism 5.0 software, San Diego, CA, USA) for curve fitting. EROD activity data measured in chicken, ring-necked pheasant and Japanese quail embryo hepatocytes were fit to a modified Gaussian curve as explained elsewhere (Kennedy et al., 1993). For each chemical, three EROD curves were generated from three separate cell culture plates. The TCDD and PCB concentrations that elicited a response equal to 20, 50 and 80% of the TCDD maximal response in a given species (TCDD<sub>20</sub>, TCDD<sub>50</sub> and TCDD<sub>80</sub>), EC<sub>50</sub> and

maximal response values are presented as the mean value of replicates obtained from three 48-well plates  $\pm$  standard error (SE). EC<sub>threshold</sub> values, which represent the concentration of a compound that elicited a significantly greater EROD response than DMSO-treated cells, were determined from concentration–response curves averaged from 3 replicate curves using a one-way analysis of variance (ANOVA) ( $p < 0.05$ ) with a Dunnett's post-hoc test ( $p < 0.05$ ). Only the concentrations that elicited a response less than 20% of the TCDD maximal response were included in the statistical analysis.

CYP1A4/5 mRNA expression data measured in chicken and Japanese quail embryo hepatocytes were fitted to a four-parameter logistic model as described previously (Head and Kennedy, 2007b). The equation integrates the hillslope, EC<sub>50</sub>, baseline response and maximal response as parameters. A single curve fit was generated for induction of CYP1A4/5 mRNA expression using data from the average of three wells from the same cell culture plate, assessed in duplicate. EC<sub>50</sub>  $\pm$  SE, TCDD<sub>20</sub>, TCDD<sub>50</sub>, TCDD<sub>80</sub> and maximal responses  $\pm$  SE were calculated from the curve fit. EC<sub>threshold</sub> values for CYP1A4/5 mRNA expression were determined from a single concentration–response curve using a one-way ANOVA ( $p < 0.05$ ) with a Dunnett's post-hoc test ( $p < 0.05$ ). Only the concentrations that elicited a response less than 20% of the TCDD maximal response were included in the statistical analysis.

Statistical differences among EC<sub>50</sub>, TCDD<sub>20</sub>, TCDD<sub>50</sub>, TCDD<sub>80</sub> and maximal response values for EROD activity or CYP1A4/5 mRNA expression were determined by performing a one-way ANOVA ( $p < 0.05$ ) followed by Tukey's post-hoc test ( $p < 0.05$ ).

#### Calculation of relative sensitivity (ReS) and relative potency (ReP)

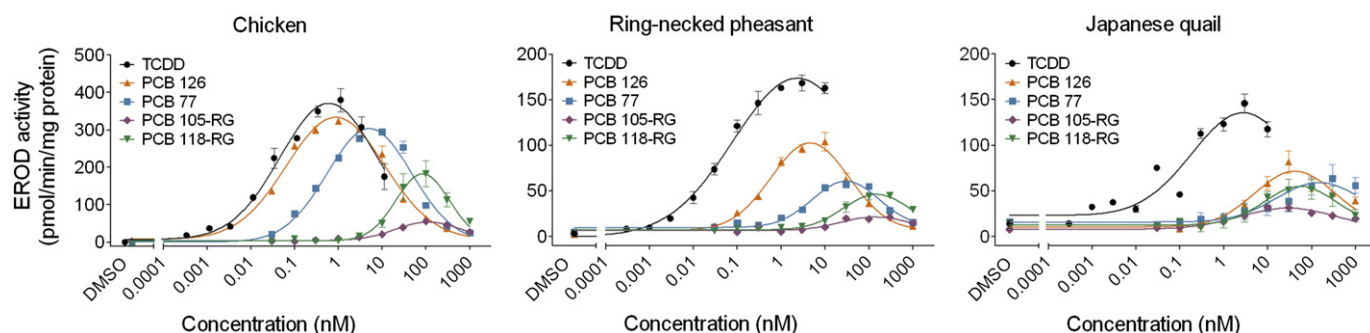
Since the chicken is considered to be the most sensitive avian species to the toxic and biochemical effects of DLCs (Head et al., 2008; Hervé et al., 2010), the sensitivity of the ring-necked pheasant and Japanese quail were expressed relative to chicken for EROD activity and CYP1A4/5 mRNA expression. Full concentration–response curves were not always obtained, and in these cases an EC<sub>50</sub> value could not be calculated. Therefore, EC<sub>threshold</sub> values were used to calculate ReS values for each species and endpoint measured. For concentration–response curves that did achieve a maximal response, EC<sub>50</sub>-based ReS values were also calculated for comparison to EC<sub>threshold</sub>-based ReS values (Table S1). ReS values were calculated as follows: EC<sub>50</sub> or EC<sub>threshold</sub> of compound  $x$  in chicken  $\div$  EC<sub>50</sub> or EC<sub>threshold</sub> of compound  $x$  in species of interest.

The maximal responses and slopes of concentration–response curves differed among DLC treatments. Therefore, the potency of PCBs was measured relative to TCDD using the systematic framework proposed by Villeneuve et al. (2000) with some modifications. ReP values based on TCDD<sub>20</sub>, TCDD<sub>50</sub> and TCDD<sub>80</sub> values obtained from the Gaussian curves or four parameter logistic models (ReP<sub>TCDD20</sub>, ReP<sub>TCDD50</sub> and ReP<sub>TCDD80</sub>) were calculated. If the maximal response or highest observed response of the PCB congener was less than 80% of the TCDD maximal response, a ReP estimate corresponding to the highest observed response for that PCB congener (ReP<sub>TCDDmax</sub>) was also calculated. If the highest observed response was less than 20% of the TCDD maximal response, a ReP was calculated based on EC<sub>threshold</sub> values. ReP values were calculated as follows: EC<sub>50</sub>, TCDD<sub>20</sub>, TCDD<sub>50</sub>, TCDD<sub>80</sub>, TCDD<sub>max</sub> or EC<sub>threshold</sub> of TCDD in species  $x \div$  EC<sub>50</sub>, TCDD<sub>20</sub>, TCDD<sub>50</sub>, TCDD<sub>80</sub>, TCDD<sub>max</sub> or EC<sub>threshold</sub> of the DLC congener of interest in species  $x$ .

## Results and discussion

#### Concentration-dependent effects of TCDD and PCBs on CYP1A induction

All DLCs significantly induced EROD activity in a concentration-dependent manner in chicken, ring-necked pheasant and Japanese quail embryo hepatocytes (Figs. 1 and 2). Maximal EROD activity was followed by a decrease in activity at higher DLC concentrations, but this decrease



**Fig. 1.** Concentration-dependent effects of TCDD, PCB 126, PCB 77, PCB 105-RG and PCB 118-RG on EROD activity in chicken, ring-necked pheasant and Japanese quail embryo hepatocyte cultures exposed for 24 h. Points represent mean EROD activity obtained from 3 replicate cell culture plates  $\pm$  standard error. Each plate received triplicate doses of each concentration of TCDD, PCB 126, PCB 77, PCB 105-RG and PCB 118-RG.

was not due to cytotoxicity, as measured by the Calcein-AM assay (data not shown).  $EC_{50}$ ,  $EC_{threshold}$ , TCDD values and maximal responses associated with each EROD curve are presented in Table 1. Significant differences in  $EC_{50}$ , TCDD values and maximal EROD activity among DLC treatments are also indicated.

CYP1A4 and CYP1A5 mRNA were induced in a concentration-dependent manner by all DLCs tested in chicken and Japanese quail embryo hepatocytes (Figs. 3 and 4). There were no significant differences in  $\beta$ -actin mRNA expression among hepatocytes treated with graded concentrations of TCDD or PCBs, indicating that changes in CYP1A4/5 mRNA expression were not a result of altered  $\beta$ -actin expression.  $EC_{50}$ ,  $EC_{threshold}$ , TCDD values and maximal responses associated with each curve are presented in Table 2.

Significant differences in maximal EROD activity and CYP1A4/5 mRNA expression were observed both among DLCs in a given species and among species exposed to a given DLC (Tables 1 and 2, Figs. 1 and 3). DLCs may induce different CYP1A responses due to ligand-specific differences in AHR conformation, resulting in altered co-activator recruitment and interactions with xenobiotic response elements (XREs) (Denison et al., 2011; Hestermann et al., 2000; Zhou et al., 2003). However, differences in cell uptake or metabolism could also contribute to differences in maximal CYP1A responses among DLCs (Hestermann et al., 2000). Interspecies variation in CYP1A expression may be due to differences in the AHR amino acid sequence, leading to differences in ligand-receptor conformation (Abnet et al., 1999; Zhou et al., 2003). Alternatively, interspecies differences in CYP1A catalytic activity (Darwish et al., 2010; Martignoni et al., 2006) could explain differences in maximal EROD activity among species.

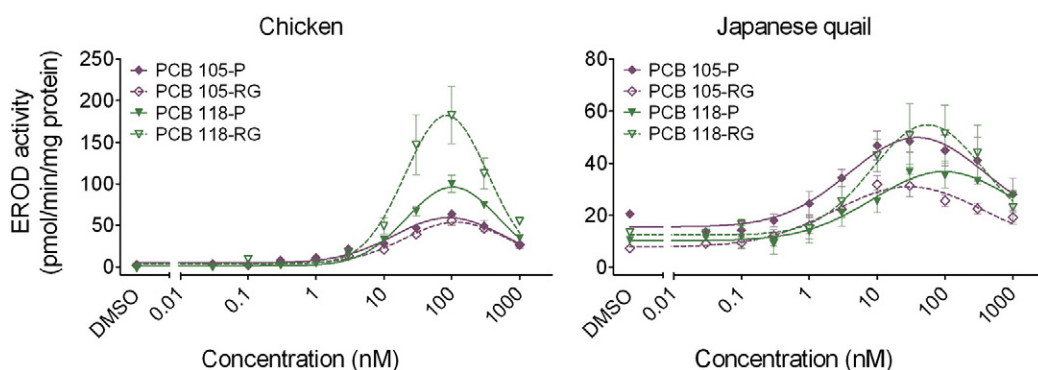
#### Relative species sensitivity to CYP1A induction by DLCs

$EC_{threshold}$ -based ReS values ( $ReS_{thr}$ ) indicated that the chicken was the most sensitive species, the ring-necked pheasant was moderately

sensitive and the Japanese quail was the least sensitive to induction of EROD activity by TCDD, PCB 126 and PCB 77 (Table 3). Similar trends were observed from  $EC_{50}$ -based ReS values.  $EC_{50}$  values for PCB 126 and 77 were significantly different among the three species (Table S1). The  $EC_{50}$  value for TCDD in chicken hepatocytes was significantly lower than that in Japanese quail hepatocytes; however the chicken and ring-necked pheasant  $EC_{50}$ s for TCDD were not significantly different.  $ReS_{thr}$  values for CYP1A4/5 mRNA also indicated that Japanese quail were less sensitive than the chicken to TCDD, PCB 126 and PCB 77 (Table 3, Table S1). Similar results were obtained from  $EC_{50}$ -based ReS values; however, an  $EC_{50}$  could not be determined in Japanese quail embryo hepatocytes exposed to PCB 77, since no maximal response for CYP1A4/5 mRNA expression was achieved.

The ring-necked pheasant and Japanese quail were equally sensitive or more sensitive to PCB 105-P, 105-RG, 118-P or 118-RG than the chicken based on  $ReS_{thr}$  values for EROD activity (Table 3). No differences were observed between EROD  $EC_{50}$  values for chicken and ring-necked pheasant hepatocytes dosed with PCB 105-RG or 118-RG, while  $EC_{50}$  values for PCB 105-RG, 118-P and 118-RG in Japanese quail hepatocytes were significantly lower than those measured in chicken and ring-necked pheasant hepatocytes (Table S1). The chicken and Japanese quail had similar sensitivities to induction of CYP1A4/5 mRNA expression by PCB 105-P, 105-RG, 118-P and 118-RG based on  $ReS_{thr}$  values and  $EC_{50}$ -based ReS values (Table 3, Table S1).

Avian species sensitivity to dioxins, furans and PCBs has been related to the identity of amino acids at positions 324 and 380 within the AHR1 ligand binding domain (Cohen-Barnhouse et al., 2011; Farmahin et al., 2012; Head et al., 2008; Hervé et al., 2010; Karchner et al., 2006; Manning et al., 2012). The results of AHR1 ligand binding domain sequencing for 86 avian species indicate that birds can be divided into one of 3 main groups based on the amino acids at these sites – type 1: Ile324\_Ser380, type 2: Ile324\_Alal380 and type 3: Val324\_Alal380 (Head et al., 2008; Farmahin et al., in press). ReS values for ring-necked pheasant



**Fig. 2.** Concentration-dependent effects of reagent-grade (RG) versus purified (P) solutions of PCB 105 and PCB 118 on EROD activity in chicken and Japanese quail embryo hepatocyte cultures exposed for 24 h. Points represent mean EROD activity obtained from 3 replicate cell culture plates  $\pm$  standard error. Each plate received triplicate doses of each PCB concentration.

**Table 1**  
Endpoints determined for EROD activity in chicken, ring-necked pheasant and Japanese quail embryo hepatocyte cultures exposed to TCDD, PCB 126, PCB 77, PCB 105-P, PCB 105-RG, PCB 118-P and PCB 118-RG. EC<sub>50</sub>, TCDD<sub>20</sub>, TCDD<sub>50</sub>, TCDD<sub>80</sub>, TCDD<sub>max</sub> and maximal responses represent the average of three replicates ± standard error (SE). EC<sub>threshold</sub> (EC<sub>thr</sub>) values were determined from a single concentration–response curve averaged from 3 replicate curves using a one-way ANOVA ( $p < 0.05$ ) followed by Dunnett's test ( $p < 0.05$ ).

Species	Compound	EC <sub>50</sub> ± SE (nM)	EC <sub>thr</sub> (nM)	TCDD <sub>20</sub> ± SE* (nM)	TCDD <sub>50</sub> ± SE* (nM)	TCDD <sub>80</sub> ± SE* (nM)	TCDD <sub>max</sub> ± SE (nM)	Maximal response ± SE (pmol/min/mg protein)
Chicken	TCDD	0.027 ± 0.004 <sup>a</sup>	0.00030	0.0058 ± 0.0008 <sup>a</sup>	0.028 ± 0.004 <sup>a</sup>	0.10 ± 0.01 <sup>a</sup>	N/A	373 ± 14 <sup>a</sup>
	PCB 126	0.043 ± 0.001 <sup>a</sup>	0.030	0.010 ± 0.0004 <sup>a</sup>	0.055 ± 0.001 <sup>b</sup>	0.26 ± 0.01 <sup>b</sup>	N/A	335 ± 4 <sup>a</sup>
	PCB 77	0.38 ± 0.04 <sup>b</sup>	0.030	0.12 ± 0.01 <sup>b</sup>	0.56 ± 0.04 <sup>c</sup>	2.85 ± 0.12 <sup>c</sup>	N/A	304 ± 2 <sup>a</sup>
	PCB 105-P	10.7 ± 2.3 <sup>c</sup>	3.00				N/A	59.8 ± 4.4 <sup>b</sup>
	PCB 105-RG	15.2 ± 0.4 <sup>c</sup>	10.0				N/A	53.8 ± 4.3 <sup>b</sup>
	PCB 118-P	16.5 ± 1.1 <sup>c</sup>	3.00	37.7 ± 9.0 <sup>c</sup>			55.1 ± 14	96.4 ± 9.3 <sup>b</sup>
	PCB 118-RG	16.6 ± 1.1 <sup>c</sup>	10.0	16.7 ± 6.0 <sup>c</sup>	44.6 ± 16 <sup>d</sup>		40.8 ± 13	183 ± 39 <sup>c</sup>
Ring-necked pheasant	TCDD	0.047 ± 0.007 <sup>a</sup>	0.0030	0.0063 ± 0.002 <sup>a</sup>	0.050 ± 0.02 <sup>a</sup>	0.31 ± 0.1	N/A	174 ± 7 <sup>a</sup>
	PCB 126	0.43 ± 0.06 <sup>b</sup>	0.10	0.26 ± 0.02 <sup>b</sup>	1.73 ± 0.18 <sup>b</sup>		2.77 ± 0.60	104 ± 5 <sup>b</sup>
	PCB 77	3.77 ± 0.20 <sup>c</sup>	1.00	6.88 ± 1.0 <sup>c</sup>			16.6 ± 4.2	60.6 ± 3.0 <sup>c</sup>
	PCB 105-RG	17.4 ± 2.2 <sup>d</sup>	30.0				N/A	21.4 ± 2.4 <sup>d</sup>
	PCB 118-RG	20.5 ± 4.1 <sup>d</sup>	10.0	70.3 ± 16 <sup>d</sup>			88.0 ± 32.8	46.6 ± 2.5 <sup>c</sup>
Japanese quail	TCDD	0.13 ± 0.04 <sup>a</sup>	0.030	0.028 ± 0.01 <sup>a</sup>	0.13 ± 0.03 <sup>a</sup>	0.60 ± 0.2	N/A	137 ± 10 <sup>a</sup>
	PCB 126	3.79 ± 0.73 <sup>b,c</sup>	1.00	2.69 ± 0.62 <sup>b</sup>	10.5 ± 0.7 <sup>b</sup>		13.4 ± 0.2	73.2 ± 11 <sup>b</sup>
	PCB 77	11.2 ± 1.2 <sup>b</sup>	100	15.6 ± 5.0 <sup>b,c</sup>			63.3 ± 26	59.0 ± 12 <sup>b,c</sup>
	PCB 105-P	4.13 ± 1.5 <sup>b,c</sup>	3.00	5.95 ± 1.8 <sup>b,c</sup>			24.9 ± 14	53.6 ± 3.2 <sup>b,c</sup>
	PCB 105-RG	1.66 ± 0.52 <sup>c</sup>	1.00	14.8 ± 7.6 <sup>b,c</sup>			24.2 ± 15	31.2 ± 1.7 <sup>c</sup>
	PCB 118-P	6.57 ± 1.4 <sup>b</sup>	3.00	36.0 ± 16 <sup>c</sup>			37.2 ± 8.7	37.3 ± 3.0 <sup>b,c</sup>
	PCB 118-RG	6.38 ± 0.86 <sup>b</sup>	10.0	9.01 ± 3.2 <sup>b,c</sup>			18.3 ± 12	54.9 ± 11 <sup>b,c</sup>

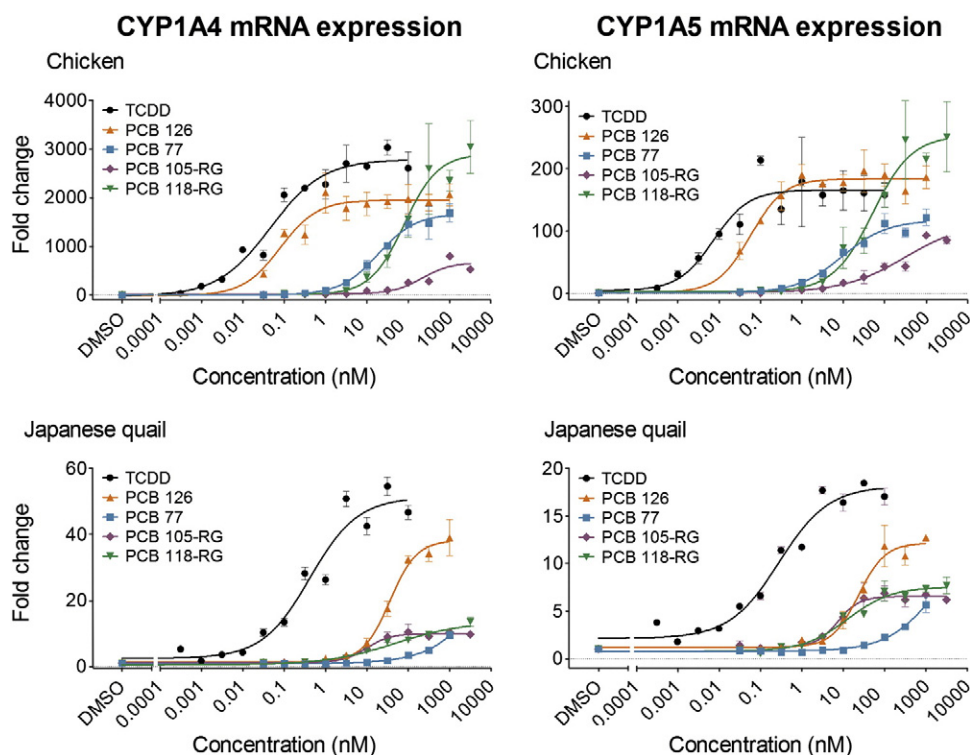
Superscript letters indicate significant differences among treatments ( $p < 0.05$ ) within each species. If two values for a given species are significantly different, those values display different superscript letters.

N/A: not applicable since response was either above 80% or below 20% of the TCDD maximal response.

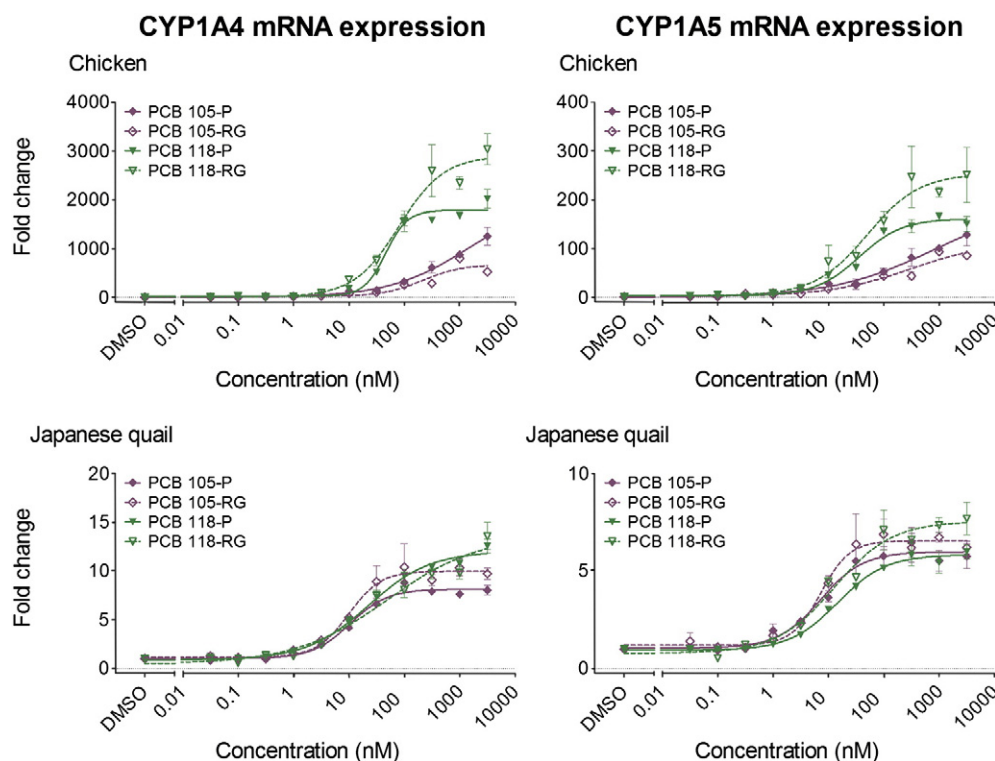
\* TCDD<sub>20</sub>, TCDD<sub>50</sub>, and TCDD<sub>80</sub> represent the TCDD and PCB concentrations that elicited a response equal to 20, 50 and 80% of the TCDD maximal response.

and Japanese quail embryo hepatocytes exposed to TCDD, PCB 126 and PCB 77 resembled ReS values obtained for *in ovo* toxicity in other type 2 and type 3 species, respectively (Head et al., 2008). Thus, the results presented herein support previous findings that type 1 species (e.g. chicken) are the most sensitive to CYP1A induction by TCDD, PCB 126 and PCB 77, type 2 species (e.g. ring-necked pheasant) are moderately sensitive and type 3 species (e.g. Japanese quail) are the least sensitive (Head et al., 2008; Manning et al., 2012). However, as was previously observed

in avian embryo hepatocytes dosed with PeCDF and hexachlorobenzene (Hervé et al., 2010; Mundy et al., 2010, 2012), the ring-necked pheasant and Japanese quail were equally sensitive or more sensitive than the chicken to CYP1A induction by PCB 105 and 118. These results are in agreement with those of a previous study where EROD-based ReS values for PCB 105 and 118 in ring-necked pheasant, turkey (*Meleagris gallopavo*) (type 2 species) and duck (*Anas platyrhynchos*) (type 3 species) hepatocyte cultures were similar to those calculated for chicken



**Fig. 3.** Concentration-dependent effects of TCDD, PCB 126, PCB 77, PCB 105-RG and PCB 118-RG on CYP1A4/5 mRNA expression in chicken and Japanese quail hepatocyte cultures exposed for 24 h. Points represent mean fold change of three replicate wells from the same cell culture plate, assessed in duplicate ± standard error.



**Fig. 4.** Concentration-dependent effects of reagent-grade (RG) versus purified (P) solutions of PCB 105 and PCB 118 on CYP1A4/5 mRNA expression in chicken and Japanese quail embryo hepatocyte cultures exposed for 24 h. Points represent mean fold change of three replicate wells from the same cell culture plate, assessed in duplicate  $\pm$  standard error.

**Table 2**

Endpoints determined from CYP1A4/5 mRNA expression in chicken and Japanese quail embryo hepatocyte cultures exposed to TCDD, PCB 126, PCB 77, PCB 105-P, PCB 105-RG, PCB 118-P and PCB 118-RG for 24 h.  $EC_{50} \pm$  standard error (SE),  $TCDD_{20}$ ,  $TCDD_{50}$ ,  $TCDD_{80}$ ,  $TCDD_{max}$  and maximal responses  $\pm$  SE were calculated from the curve fit.  $EC_{threshold}$  ( $EC_{thr}$ ) values were calculated using a one-way ANOVA ( $p < 0.05$ ) followed by Dunnett's test ( $p < 0.05$ ).

Species	Compound	Endpoint	EC <sub>50</sub> ±SE (nM)	EC <sub>thr</sub> (nM)	TCDD <sub>20</sub> <sup>*</sup> (nM)	TCDD <sub>50</sub> <sup>*</sup> (nM)	TCDD <sub>80</sub> <sup>*</sup> (nM)	TCDD <sub>max</sub> (nM)	Maximal response ± SE (fold change)
Chicken	TCDD	CYP1A4	0.046 ± 1.4 <sup>a</sup>	0.0010	0.0064	0.047	0.36	N/A	2780 ± 130 <sup>a</sup>
	PCB 126	CYP1A4	0.080 ± 1.4 <sup>a</sup>	0.030	0.032	0.21	6.43	1950 ± 66 <sup>a,b</sup>	
	PCB 77	CYP1A4	18.6 ± 1.2 <sup>b</sup>	0.30	9.0	99.4	10,000	1670 ± 76 <sup>a,b</sup>	
	PCB 105-P	CYP1A4	1490 ± 3 <sup>c</sup>	3.00	295	5301	1,200,000	2040 ± 710 <sup>a,b</sup>	
	PCB 105-RG	CYP1A4	215 ± 1 <sup>b,c</sup>	10.0	766		64,500	669 ± 72 <sup>b</sup>	
	PCB 118-P	CYP1A4	46.1 ± 1.1 <sup>b</sup>	10.0	31.7	80.8	949	1780 ± 52 <sup>a,b</sup>	
	PCB 118-RG	CYP1A4	80.1 ± 1.3 <sup>b</sup>	10.0	20.4	73.8	246	2900 ± 200 <sup>a</sup>	
Japanese quail	TCDD	CYP1A4	0.42 ± 1.3 <sup>a</sup>	0.0030	0.055	0.39	2.54	N/A	51.3 ± 2.6 <sup>a</sup>
	PCB 126	CYP1A4	35.8 ± 1.2 <sup>b,c</sup>	3.00	16.6	61.1	18,200	38.2 ± 1.8 <sup>b</sup>	
	PCB 77	CYP1A4	NC	100				N/A	9.47 ± 1.9 <sup>f,c</sup>
	PCB 105-P	CYP1A4	10.5 ± 1.1 <sup>b</sup>	3.00				N/A	8.15 ± 0.18 <sup>c</sup>
	PCB 105-RG	CYP1A4	9.84 ± 1.3 <sup>b</sup>	10.0				N/A	9.95 ± 0.45 <sup>c</sup>
	PCB 118-P	CYP1A4	22.3 ± 1.2 <sup>b,c</sup>	10.0	477		1340	12.0 ± 0.4 <sup>c</sup>	
	PCB 118-RG	CYP1A4	49.8 ± 1.7 <sup>c</sup>	10.0	871		119,000	13.6 ± 1.5 <sup>c</sup>	
Chicken	TCDD	CYP1A5	0.0072 ± 1.8 <sup>a</sup>	0.0010	0.0017	0.0068	0.025	N/A	166 ± 11 <sup>a</sup>
	PCB 126	CYP1A5	0.057 ± 1.4 <sup>a</sup>	0.030	0.014	0.047	0.14	N/A	184 ± 7 <sup>a</sup>
	PCB 77	CYP1A5	9.31 ± 1.3 <sup>b</sup>	1.00	3.0	28.6	2000	118 ± 7 <sup>a</sup>	
	PCB 105-P	CYP1A5	842 ± 6 <sup>c</sup>	1.00	29.5	432	3911	N/A	197 ± 79 <sup>a</sup>
	PCB 105-RG	CYP1A5	309 ± 2 <sup>b,c</sup>	3.00	64.3	1559	327,000	115 ± 25 <sup>a</sup>	
	PCB 118-P	CYP1A5	35.7 ± 1.2 <sup>b,c</sup>	1.00	9.82	36.5	139	N/A	162 ± 6 <sup>a</sup>
	PCB 118-RG	CYP1A5	50.4 ± 1.6 <sup>b,c</sup>	1.00	5.81	22.0	54.4	N/A	254 ± 27 <sup>a</sup>
Japanese quail	TCDD	CYP1A5	0.28 ± 1.3 <sup>a</sup>	0.0030	0.024	0.23	1.71	N/A	18.2 ± 0.6 <sup>a</sup>
	PCB 126	CYP1A5	23.5 ± 1.3 <sup>b</sup>	10.0	12.3	56.3	4350	12.2 ± 0.7 <sup>b</sup>	
	PCB 77	CYP1A5	NC	100	553		995	5.66 ± 0.83 <sup>f,c</sup>	
	PCB 105-P	CYP1A5	7.12 ± 1.3 <sup>c</sup>	1.00	14.4		28,400	5.97 ± 0.22 <sup>c</sup>	
	PCB 105-RG	CYP1A5	7.32 ± 1.3 <sup>c</sup>	3.00	9.43		114	6.54 ± 0.27 <sup>c</sup>	
	PCB 118-P	CYP1A5	14.7 ± 1.2 <sup>b,c</sup>	3.00	37.3		4180	5.82 ± 0.17 <sup>c</sup>	
	PCB 118-RG	CYP1A5	12.8 ± 1.3 <sup>b,c</sup>	10.0	24.9		23,300	7.54 ± 0.36 <sup>c</sup>	

Superscript letters indicate significant differences among treatments ( $p < 0.05$ ) within each species. If two values for a given species are significantly different, those values display different superscript letters.

N/A: not applicable since response was either above 80% or below 20% of the TCDD maximal response.

NC:  $EC_{50}$  not calculated since maximal response was not reached.

\*  $TCDD_{20}$ ,  $TCDD_{50}$ , and  $TCDD_{80}$  represent the TCDD and PCB concentrations that elicited a response equal to 20, 50 and 80% of the TCDD maximal response.

† No maximal response was reached. Values represent highest observed response.

**Table 3**

Relative sensitivity (ReS) and relative potency (ReP) values obtained from EROD activity, CYP1A4/5 mRNA expression and luciferase reporter gene (LRG) activity.  $EC_{\text{threshold}}$ -based ReS values ( $ReS_{\text{thr}}$ ) for the ring-necked pheasant and Japanese quail were calculated relative to the chicken. Average relative potency values ( $ReP_{\text{avg}}$ ) for PCB 126, 77, 105 and 118 relative to TCDD were calculated from  $EC_{\text{threshold}}$ -,  $TCDD_{20}$ -,  $TCDD_{50}$ -,  $TCDD_{80}$ - and  $TCDD_{\text{max}}$ -based ReP values.

Species	Compound	$ReS_{\text{thr}}$				$ReP_{\text{avg}}$			
		EROD	CYP1A4	CYP1A5	LRG <sup>a</sup>	EROD	CYP1A4	CYP1A5	LRG <sup>a</sup>
Chicken	TCDD	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	PCB 126	1.00	1.00	1.00	1.00	0.36	0.12	0.12	0.074
	PCB 77	1.00	1.00	1.00	1.00	0.035	0.0011	0.00046	0.0021
	PCB 105-P	1.00	1.00	1.00	1.00	0.00010	0.000091	0.00027	0.000030
	PCB 105-RG	1.00	1.00	1.00	1.00	0.000030	0.000036	0.000091	0.000030
	PCB 118-P	1.00	1.00	1.00	1.00	0.0010	0.00025	0.00039	0.000062
	PCB 118-RG	1.00	1.00	1.00	1.00	0.00041	0.00063	0.00052	0.000069
Ring-necked pheasant	TCDD	0.10			0.10	1.00			1.00
	PCB 126	0.30			0.030	0.022			0.037
	PCB 77	0.030			0.033	0.00085			0.0010
	PCB 105-RG	0.33			<0.33	0.000033			<0.00010
	PCB 118-RG	1.00			<0.10	0.000092			<0.00010
Japanese quail	TCDD	0.010	0.33	0.33	0.0030	1.00	1.00	1.00	1.00
	PCB 126	0.010	0.010	0.0030	0.030	0.016	0.0027	0.0016	0.32
	PCB 77	0.0010	0.0030	0.010	0.0048	0.0010	0.000030	0.000041	0.0048
	PCB 105-P	0.30	1.00	1.00	<0.33	0.0057	0.0010	0.0016	<0.0033
	PCB 105-RG	10.00	1.00	1.00	<0.33	0.011	0.00030	0.0014	<0.0033
	PCB 118-P	1.00	1.00	0.33	0.10	0.0039	0.00015	0.00055	0.0033
	PCB 118-RG	1.00	1.00	0.10	0.10	0.0030	0.00012	0.00043	0.0033

<sup>a</sup> LRG assay ReS and ReP values from Manning et al. (2012).

hepatocyte cultures (Kennedy et al., 1996). Ligand-dependent differences in binding with the different AHR1 types could explain such reversals in relative species sensitivity and could be determined by AHR1 binding assays or homology modeling studies.

#### Relative PCB potency in chicken, ring-necked pheasant and Japanese quail hepatocyte cultures

Based on average ReP values ( $ReP_{\text{avg}}$ ) (Table 3), visual inspection of concentration–response curves (Figs. 1 and 2) and the results of statistical analyses performed among EROD endpoints for different DLC treatments in a given species (Table 1), the rank order of DLC potency was:  $TCDD \geq PCB 126 > PCB 77 > PCB 118-P \approx PCB 118-RG \geq PCB 105-P \approx PCB 105-RG$  in chicken hepatocytes,  $TCDD > PCB 126 > PCB 77 > PCB 118-RG \geq PCB 105-RG$  in ring-necked pheasant hepatocytes and  $TCDD > PCB 126 \geq PCB 105-RG \approx PCB 105-P \approx PCB 118-RG \approx PCB 118-P \approx PCB 77$  in Japanese quail hepatocytes. The rank order of DLC potency for CYP1A4/5 mRNA expression based on  $ReP_{\text{avg}}$  values (Table 3), visual inspection of concentration–response curves (Figs. 3 and 4) and the results of statistical analyses performed among mRNA expression endpoints for different DLC treatments in a given species (Table 2) was  $TCDD \geq PCB 126 > PCB 77 \geq PCB 118-RG \approx PCB 118-P \geq PCB 105-P \approx PCB 105-RG$  in chicken hepatocytes and  $TCDD > PCB 126 \geq PCB 105-P \approx PCB 105-RG \geq PCB 118-P \approx PCB 118-RG > PCB 77$  in Japanese quail hepatocytes.  $ReP_{\text{avg}}$  values were calculated from  $EC_{\text{threshold}}$ -,  $TCDD_{20}$ -,  $TCDD_{50}$ -,  $TCDD_{80}$ - and  $TCDD_{\text{max}}$ -based ReP values.  $EC_{50}$ -based ReP values were excluded from the  $ReP_{\text{avg}}$  calculation since these values can often overestimate the potency of PCBs with lower maximal responses than TCDD (Kennedy et al., 1996).

The rank order of DLC potency and the ReP values obtained from EROD and QPCR assays in chicken hepatocytes were generally similar to those suggested by the WHO TEFs for birds (van den Berg et al., 1998). However, the PCB 118 ReP was 1 to 2 orders of magnitude greater than its WHO TEF of 0.00001, and therefore had approximately the same potency as PCB 105. This finding is consistent with the results of previous EROD studies where PCB 118 and PCB 105 were observed to be equally potent in avian hepatocyte cultures from several species (Kennedy et al., 1996). QPCR-based RePs for PCB 77 in chicken embryo hepatocytes were 50 to 100 times lower than the WHO TEF value of 0.05, however, this difference was not observed between the EROD-based ReP and TEF. QPCR-based RePs for PCB 77 were also 3 orders of magnitude lower

than the corresponding EROD-based ReP in Japanese quail embryo hepatocytes. This could be due to higher degradation rates for CYP1A mRNA relative to CYP1A protein levels and CYP1A activity, which were previously observed in Sprague-Dawley rats, mummichog (*Fundulus heteroclitus*) and striped bass (*Morone saxatilis*) exposed to  $\beta$ -naphthoflavone (BNF) (Chen et al., 2010; Durieux et al., 2012; Kloepper-Sams and Stegeman, 1989). Treatment-related differences in CYP1A1 mRNA and protein level profiles were also observed in rainbow trout hepatocytes exposed to BNF and TCDD and were attributed to increased metabolism and inactivation of BNF, relative to TCDD (Pesonen et al., 1992). Since PCB 77 is more easily metabolized than other DLCs, such as TCDD and PCB 126 (Bastien et al., 1997), mRNA levels likely decline at a faster rate, which could explain why differences between EROD and QPCR-based ReP values are not observed to the same extent with the other PCBs tested.

EROD and QPCR-based RePs for PCB 126 in the ring-necked pheasant and Japanese quail were between 1 and 2 orders of magnitude lower than the chicken ReP (Table 3) and WHO TEF value of 0.1.  $LD_{50}$ -based ReP values for PCB 126 were also lower in the double-crested cormorant (*Phalacrocorax auritus*; type 3) than in the chicken; however the ReP values for each of these species were within one order of magnitude of each other (Farmahin et al., in press; Powell et al., 1996, 1998). EROD-based RePs for PCB 77 in the ring-necked pheasant and Japanese quail were 1 to 2 orders of magnitude lower than the chicken ReP (Table 3) and the WHO TEF value of 0.05. The lower PCB 126 and 77 RePs observed in the ring-necked pheasant and Japanese quail indicate that the WHO TEFs may overestimate the potency of PCB 126 and 77 in these species and other type 2 and type 3 species of birds. Although the TEQ concentrations observed in tree swallow (*Tachycineta bicolor*; type 2) eggs along the Hudson River of New York, USA (1.73–12.7 ng/g TEQ) were up to 10 times greater than the TCDD  $LD_{50}$  of 1.2 ng/g in ring-necked pheasant embryos (Cohen-Barnhouse et al., 2011; Secord et al., 1999), only slight effects on reproductive success and parental behavior were observed (McCarty and Secord, 1999). Since PCB 77 contributed 82–87% of the TEQs measured in tree swallow eggs and nestlings (Secord et al., 1999), overestimation of the PCB 77 TEF by 1 to 2 orders of magnitude in type 2 species could explain the lack of significant embryolethal effects in tree swallows along the Hudson River.

While PCB 126 and 77 RePs in ring-necked pheasant and Japanese quail hepatocytes were below the WHO TEFs, EROD- and QPCR-based RePs for PCB 105 in Japanese quail hepatocytes were 1 to 2 orders of magnitude greater than their corresponding TEF value of 0.0001 and

the RePs for PCB 105 in chicken hepatocytes (Table 3). PCB 118 RePs in chicken, ring-necked pheasant and Japanese quail embryo hepatocytes were also 1 to 2 orders of magnitude greater than the PCB 118 TEF. These *in vitro* results indicate that the current TEFs for birds may underestimate the relative potency of PCB 105 in Japanese quail and other type 3 species and the relative potency of PCB 118 in most avian species. Additional egg injection studies with PCB 105 and 118 could be performed to test whether this is also the case *in ovo*. The potency of PCBs relative to TCDD varies significantly between the chicken, ring-necked pheasant and Japanese quail, and is likely a result of differences in relative species sensitivity to different PCBs. Therefore, the application of one set of TEF values for calculating TEQs in all species of birds may not be appropriate.

#### Effects of contamination on RePs for mono-ortho PCBs

A previous study that investigated the effects of several purified mono-ortho PCBs on AHR-dependent gene expression in mouse and rat hepatoma cell lines found that PCB 105 and 118 RePs were 50 to 100 times lower than WHO mammalian TEFs (Peters et al., 2006). The authors suggested that the differences between the ReP values obtained from their study and corresponding TEF values may have been due to contamination of mono-ortho PCB solutions with more potent AHR agonists in previous studies that were used to derive the WHO TEFs (Peters et al., 2006). We further tested this hypothesis by comparing induction of EROD activity and CYP1A4/5 mRNA expression in avian hepatocyte cultures exposed to either reagent-grade or purified PCB 105 and 118. PCB 77 was the major contaminant identified in PCB 105-P, 105-RG, 118-P and 118-RG (Manning et al., 2012). PCB 118-RG had the highest levels of PCB 77 (2470 ppm), whereas the PCB 77 concentrations found in PCB 105-P, 105-RG and 118-P were approximately 20 ppm (Manning et al., 2012).

The maximal EROD response induced by PCB 118-RG (183 pmol/min/mg protein) in chicken hepatocytes was greater than that induced by PCB 118-P (94.6 pmol/min/mg protein) (Table 1, Fig. 2) but no further differences in  $ReP_{avg}$ ,  $EC_{50}$ ,  $EC_{threshold}$  or  $TCDD_x$  values were observed between purified and reagent-grade PCBs (Tables 1 and 2, Figs. 2 and 4). Furthermore,  $ReP_{avg}$  values between reagent-grade and purified PCBs differed by a maximum of 3-fold (Table 3), indicating that contamination of mono-ortho PCB solutions did not lead to significant overestimation of their ReP values for CYP1A induction in avian hepatocyte cultures.

#### Comparisons between CYP1A induction in avian hepatocyte cultures, luciferase reporter gene (LRG) activity and *in ovo* toxicity

EROD- and CYP1A4/5 mRNA expression-based ReS and ReP values for TCDD and PCB 126, 77, 105-P, 105-RG, 118-P and 118-RG in the chicken, ring-necked pheasant and Japanese quail were compared to values previously obtained from an LRG assay measuring induction of a CYP1A5 reporter gene (Table 3) (Manning et al., 2012).  $ReS_{thr}$  values for a given species and DLC were generally within one order of magnitude between the EROD, QPCR and LRG assays. A greater range in Japanese quail  $ReS_{thr}$  values for TCDD was observed where EROD and LRG assay  $ReS_{thr}$  values were similar but were 30 to 100 times lower than the QPCR-based  $ReS_{thr}$  values. The rank order of species sensitivity obtained from EROD, QPCR and LRG assays was generally similar for TCDD, PCB 126 and PCB 77. However, ring-necked pheasant and Japanese quail AHR1 constructs exposed to PCB 105 and 118 were less sensitive than the chicken constructs in the LRG assay (Manning et al., 2012), whereas ring-necked pheasant and Japanese quail hepatocytes exposed to these PCBs were equally sensitive or more sensitive than chicken hepatocytes. Since little or no induction of luciferase activity was observed in cells transfected with type 2 and type 3 AHR1 constructs and exposed to PCB 105 and 118, the limited sensitivity of the LRG assay likely affected our ability to compare the effects of PCB 105 and 118 among AHR1 constructs.

The rank order of DLC potency observed from EROD and CYP1A4/5 mRNA expression in chicken and ring-necked pheasant hepatocyte

cultures was similar to that observed for chicken and ring-necked pheasant, as well as other type 1 and type 2 AHR1 constructs in the LRG assay (Manning et al., 2012). Although LRG assay results indicated that PCB 126 was more potent than PCB 77, 105 and 118 in the Japanese quail and other type 3 AHR1 constructs (Manning et al., 2012), the results of EROD and QPCR assays revealed that PCB 126 was approximately as potent as these congeners in Japanese quail embryo hepatocytes. Limited sensitivity of the LRG assay could have also affected ReP estimates for PCB 77, 105 and 118 since little or no luciferase activity was induced by these PCBs in type 3 constructs (Manning et al., 2012).

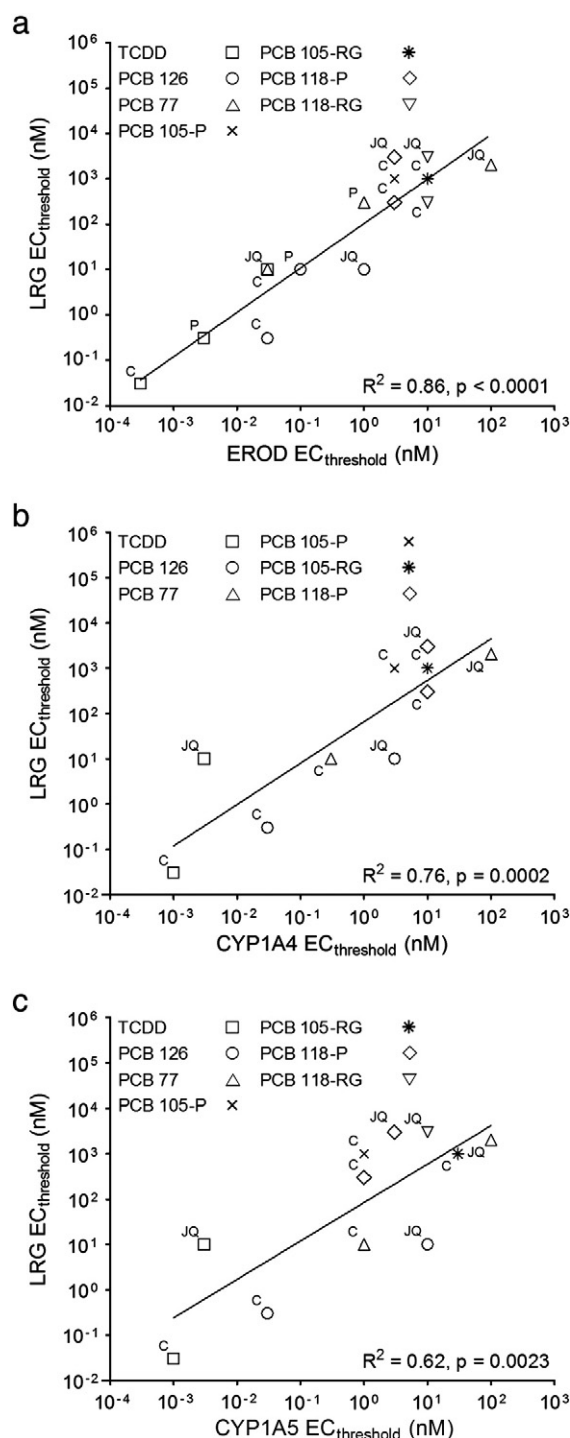
Linear regression analyses between reporter gene activity data and EROD activity or CYP1A4/5 mRNA expression data were conducted. The results of the linear regressions comparing  $EC_{threshold}$  values from the LRG, EROD and QPCR assays are presented in Fig. 5. LRG and EROD assay  $EC_{50}$ ,  $EC_{threshold}$  and  $TCDD_{20}$  values were significantly correlated ( $0.81 \leq R^2 \leq 0.92$ ,  $p \leq 0.0061$ ), whereas weaker relationships were observed between LRG activity and CYP1A4 ( $0.74 \leq R^2 \leq 0.82$ ,  $p \leq 0.0622$ ) or CYP1A5 ( $0.62 \leq R^2 \leq 0.84$ ,  $p \leq 0.0285$ ) mRNA expression (Table S3). These findings are consistent with the observation that ReS and ReP values obtained from the EROD and LRG assays were generally similar, whereas notable differences between EROD/LRG activity- and CYP1A4/5 mRNA expression-based ReS and ReP values were identified (Table 3).

Significant or near-significant relationships were also observed between *in ovo*  $LD_{50}$  data from the literature (Table S2) and  $EC_{50}$ ,  $EC_{threshold}$  and  $TCDD_{20}$  values measured for EROD activity ( $0.91 \leq R^2 \leq 0.98$ ,  $p \leq 0.0011$ ), CYP1A4 mRNA expression ( $0.76 \leq R^2 \leq 0.81$ ,  $p \leq 0.0229$ ) and CYP1A5 mRNA expression ( $0.58 \leq R^2 \leq 0.81$ ,  $p \leq 0.0766$ ) (Fig. 6, Table S4). Stronger relationships were observed between  $LD_{50}$  data and EROD activity than between  $LD_{50}$ s and CYP1A4/5 mRNA expression. LRG assay endpoints were also significantly associated with *in ovo*  $LD_{50}$  values (Manning et al., 2012), indicating that AHR1-induced enzyme activity better predicts DLC-induced embryo lethality than CYP1A4/5 mRNA expression. Although similar trends in CYP1A mRNA induction and EROD activity are often observed, factors other than mRNA induction, including mRNA stability and protein turnover, signal sensing and transduction, and post-translational modifications of proteins can, influence protein levels and activity (Regoli et al., 2011). Because proteins, rather than mRNA, are the actors in cellular reactions, measures of protein activity are more biologically relevant than mRNA expression (Heijne et al., 2005). Thus, ReS and ReP values determined from EROD and LRG activity are likely to be more representative of *in ovo* toxicity-based ReS and ReP values than those based on CYP1A4/5 mRNA expression. The EROD assay is considerably quicker and cheaper to use than QPCR and therefore, represents a more economical and useful screening tool for predicting overt toxicity of DLCs compared to QPCR.

#### General conclusions

The results obtained for EROD activity and/or CYP1A4/5 mRNA expression in chicken, ring-necked pheasant and Japanese quail embryo hepatocytes support and complement the findings previously obtained from the LRG assay (Manning et al., 2012). As predicted by the LRG assay, the chicken was the most sensitive species to induction of CYP1A by TCDD, PCB 126 and PCB 77. However, the ring-necked pheasant and Japanese quail were either equally sensitive or more sensitive than the chicken to CYP1A induction by PCB 105 and 118.

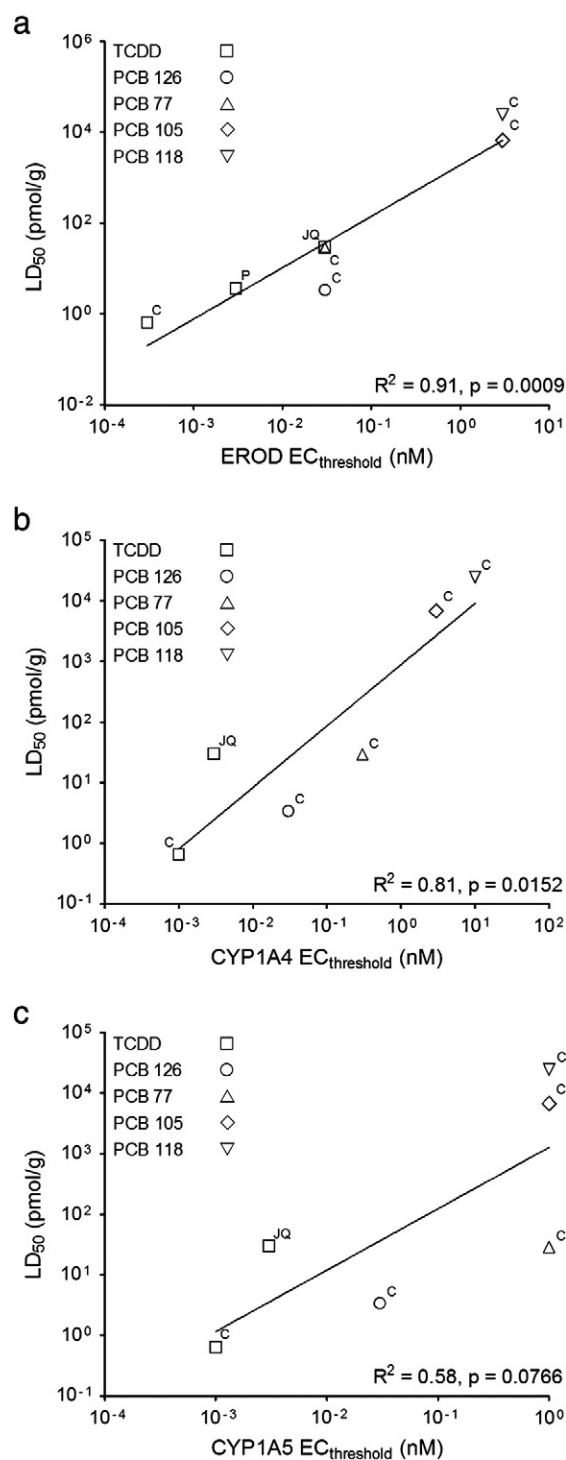
Significant differences in PCB ReP values were observed among species. While the chicken RePs generally resembled the WHO TEF values, ReP values for PCB 126 and 77 in ring-necked pheasant and Japanese quail hepatocytes were lower than those measured in chicken hepatocytes. In contrast, RePs for PCB 105 and 118 in the Japanese quail were higher than those in the chicken. These findings can be extrapolated to other avian species based on their AHR1 genotype (Manning et al., 2012; Farmahin et al., in press) and could be used, along with the results



**Fig. 5.** Correlation between  $EC_{50}$  values from a luciferase reporter gene (LRG) assay (Manning et al., 2012) and  $EC_{50}$  values for (a) EROD activity, (b) CYP1A4 mRNA expression and (c) CYP1A5 mRNA expression in the chicken (C), ring-necked pheasant (P) and Japanese quail (JQ).

of additional *in vitro* and *in vivo* studies, to develop ReP values based on a species' AHR1 genotype.

Finally, EROD activity in avian hepatocyte cultures was more strongly associated with both luciferase activity in cells transfected with avian AHR1 constructs and *in ovo* PCB toxicity than CYP1A4/5 mRNA expression. This supports the selection of the EROD assay over the QPCR assay for relating changes in CYP1A gene expression to overt toxicity of PCBs. The results presented herein support those obtained from the LRG assay (Manning et al., 2012) and indicate that AHR1-mediated induction of enzyme activity is strongly associated with PCB-induced



**Fig. 6.** Correlation between  $LD_{50}$  values obtained from the literature and  $EC_{50}$  values for (a) EROD activity (b) CYP1A4 mRNA expression and (c) CYP1A5 mRNA expression in the chicken (C), ring-necked pheasant (P) and Japanese quail (JQ).

embryolethality in several species of birds. To overcome problems associated with the limited sensitivity of the LRG assay that arise when testing the effects of weak AHR agonists, the EROD and LRG assays can be used in a complementary fashion to reduce the number of animals required for predicting DLC toxicity.

#### Conflict of interest

The authors declare that there are no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data includes EC<sub>50</sub>-based ReS values (Table S1) and *in ovo* LD<sub>50</sub> values collected from the literature for the chicken, ring-necked pheasant and Japanese quail (Table S2). Linear regression equations and associated R<sup>2</sup> and *p* values are also provided for the relationships between reporter gene activity and EROD activity or CYP1A4/5 mRNA expression (Table S3) and between *in ovo* toxicity and EROD activity or CYP1A4/5 mRNA expression (Table S4).

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.taap.2012.10.030>.

## References

- Abnet, C.C., Tanguay, R.L., Heideman, W., Peterson, R.E., 1999. Transactivation activity of human, zebrafish, and rainbow trout aryl hydrocarbon receptors expressed in COS-7 cells: greater insight into species differences in toxic potency of polychlorinated dibenzo-p-dioxin, dibenzofuran, and biphenyl congeners. *Toxicol. Appl. Pharmacol.* 159, 41–51.
- Bastien, L.J., Kennedy, S.W., Lorenzen, A., 1997. Ethoxyresorufin O-deethylase (EROD) induction by halogenated aromatic hydrocarbons (HAHs) in chicken embryo hepatocyte cultures: time-dependent effects on the dose-response curves. *Organohalogen Compd.* 34, 215–220.
- Chen, E.P., Chen, L., Ji, Y., Tai, G., Wen, Y.H., Ellens, H., 2010. A mechanism-based mathematical model of aryl hydrocarbon receptor-mediated CYP1A induction in rats using beta-naphthoflavone as a tool compound. *Drug Metab. Dispos.* 38, 2278–2285.
- Cohen-Barnhouse, A.M., Zwiernik, M.J., Link, J.E., Fitzgerald, S.D., Kennedy, S.W., Hervé, J.C., Giesy, J.P., Wiseman, S.B., Yang, Y., Jones, P.D., Wan, Y., Collins, B., Newsted, J.L., Kay, D.P., Bursian, S.J., 2011. Sensitivity of Japanese quail (*Coturnix japonica*), Common pheasant (*Phasianus colchicus*), and White Leghorn chicken (*Gallus gallus domesticus*) embryos to *in ovo* exposure to TCDD, PeCDF, and TCDF. *Toxicol. Sci.* 119, 93–103.
- Darwish, W.S., Ikenaka, Y., Eldaly, E.A., Ohno, M., Sakamoto, K.Q., Fujita, S., Ishizuka, M., 2010. Cytochrome P450 1A-dependent activities in deer, cattle and horses. *J. Vet. Med. Sci.* 72, 561–566.
- Denison, M.S., Soshilov, A.A., He, G., DeGroot, D.E., Zhao, B., 2011. Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the aryl hydrocarbon (dioxin) receptor. *Toxicol. Sci.* 124, 1–22.
- Durieux, E.D.H., Connon, R.E., Werner, I., D'Abronzio, L.S., Fitzgerald, P.S., Spearow, J.L., Ostrach, D.J., 2012. Cytochrome P4501A mRNA and protein induction in striped bass (*Morone saxatilis*). *Fish Physiol. Biochem.* 38, 1107–1116.
- Farmahin, R., Manning, G.E., Crump, D., Mundy, L.J., Jones, S.P., Hahn, M.E., Karchner, S.I., Giesy, J.P., Bursian, S.J., Zwiernick, M.J., Fredricks, T.B., Kennedy, S.W., in press. The amino acid sequence of the ligand binding domain of the aryl hydrocarbon receptor 1 (AHR1) predicts sensitivity of wild birds to effects of dioxin-like compounds. *Toxicol. Sci.* <http://dx.doi.org/10.1093/toxsci/kfs259>.
- Farmahin, R., Wu, D., Crump, D., Hervé, J.C., Jones, S.P., Hahn, M.E., Karchner, S.I., Giesy, J.P., Bursian, S.J., Zwiernick, M.J., Kennedy, S.W., 2012. Sequence and *in vitro* function of chicken, ring-necked pheasant, and Japanese quail AHR1 predict *in vivo* sensitivity to dioxins. *Environ. Sci. Technol.* 46, 2967–2975.
- Head, J.A., Kennedy, S.W., 2007a. Same-sample analysis of ethoxyresorufin-O-deethylase activity and cytochrome P4501A mRNA abundance in chicken embryo hepatocytes. *Anal. Biochem.* 360, 294–302.
- Head, J.A., Kennedy, S.W., 2007b. Differential expression, induction, and stability of CYP1A4 and CYP1A5 mRNA in chicken and herring gull embryo hepatocytes. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 145, 617–624.
- Head, J.A., Kennedy, S.W., 2010. Correlation between an *in vitro* and an *in vivo* measure of dioxin sensitivity in birds. *Ecotoxicology* 19, 377–382.
- Head, J.A., O'Brien, J.M., Kennedy, S.W., 2006. Exposure to 3,3',4,4',5-pentachlorobiphenyl during embryonic development has a minimal effect on the cytochrome P4501A response to 2,3,7,8-tetrachlorodibenzo-p-dioxin in cultured chicken embryo hepatocytes. *Environ. Toxicol. Chem.* 25, 2981–2989.
- Head, J.A., Hahn, M.E., Kennedy, S.W., 2008. Key amino acids in the aryl hydrocarbon receptor predict dioxin sensitivity in avian species. *Environ. Sci. Technol.* 42, 7535–7541.
- Heijne, W.H., Stierum, R.H., Leeman, W.R., van Ommen, B., 2005. The introduction of toxicogenomics; potential new markers of hepatotoxicity. *Cancer Biomark.* 1, 41–57.
- Hervé, J.C., Crump, D., Jones, S.P., Mundy, L.J., Giesy, J.P., Zwiernick, M.J., Bursian, S.J., Jones, P.D., Wiseman, S.B., Wan, Y., Kennedy, S.W., 2010. Cytochrome P4501A induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin and two chlorinated dibenzofurans in primary hepatocyte cultures of three avian species. *Toxicol. Sci.* 113, 380–391.
- Hestermann, E.V., Stegeman, J.J., Hahn, M.E., 2000. Relative contributions of affinity and intrinsic efficacy to aryl hydrocarbon receptor ligand potency. *Toxicol. Appl. Pharmacol.* 168, 160–172.
- Karchner, S.I., Franks, D.G., Kennedy, S.W., Hahn, M.E., 2006. The molecular basis for differential dioxin sensitivity in birds: role of the aryl hydrocarbon receptor. *Proc. Natl. Acad. Sci. U. S. A.* 103, 6252–6257.
- Kennedy, S.W., Lorenzen, A., James, C.A., Collins, B.T., 1993. Ethoxyresorufin-O-deethylase and porphyrin analysis in chicken embryo hepatocyte cultures with fluorescence multiwell plate reader. *Anal. Biochem.* 211, 102–112.
- Kennedy, S.W., Jones, S.P., Bastien, L.J., 1995. Efficient analysis of cytochrome P4501A catalytic activity, porphyrins, and total proteins in chicken embryo hepatocyte cultures with a fluorescence plate reader. *Anal. Biochem.* 226, 362–370.
- Kennedy, S.W., Lorenzen, A., Jones, S.P., Hahn, M.E., Stegeman, J.J., 1996. Cytochrome P4501A induction in avian hepatocyte cultures: a promising approach for predicting the sensitivity of avian species to toxic effects of halogenated aromatic hydrocarbons. *Toxicol. Appl. Pharmacol.* 141, 214–230.
- Kennedy, S.W., Jones, S.P., Elliott, J.E., 2003. Sensitivity of bald eagle (*Haliaeetus leucocephalus*) hepatocyte cultures to induction of cytochrome P4501A by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Ecotoxicology* 12, 163–170.
- Klopper-Sams, P.J., Stegeman, J.J., 1989. The temporal relationship between P450E protein content, catalytic activity, and mRNA levels in the teleost *Fundulus heteroclitus* following treatment with  $\beta$ -naphthoflavone. *Arch. Biochem. Biophys.* 268, 525–535.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-ΔΔC<sub>T</sub></sup> method. *Methods* 25, 402–408.
- Manning, G.E., Farmahin, R., Crump, D., Jones, S.P., Klein, J., Konstantinov, A., Potter, D., Kennedy, S.W., 2012. A luciferase reporter gene assay and aryl hydrocarbon receptor 1 genotype predict the embryolethality of polychlorinated biphenyls in avian species. *Toxicol. Appl. Pharmacol.* 263, 390–399.
- Martignoni, M., Groothuis, G.M.M., de Kanter, R., 2006. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert Opin. Drug Metab. Toxicol.* 2, 875–894.
- McCarty, J.P., Secord, A.L., 1999. Reproductive ecology of tree swallows (*Tachycineta bicolor*) with high levels of polychlorinated biphenyl contamination. *Environ. Toxicol. Chem.* 18, 1433–1439.
- Mundy, L.J., Jones, S.P., Crump, D., Hervé, J.C., Konstantinov, A., Utley, F., Potter, D., Kennedy, S.W., 2010. Highly purified hexachlorobenzene induces cytochrome P4501A in primary cultures of chicken embryo hepatocytes. *Toxicol. Appl. Pharmacol.* 248, 185–193.
- Mundy, L.J., Crump, D., Jones, S.P., Konstantinov, A., Utley, F., Potter, D., Kennedy, S.W., 2012. Induction of cytochrome P4501A by highly purified hexachlorobenzene in primary cultures of ring-necked pheasant and Japanese quail embryo hepatocytes. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 155, 498–505.
- Okey, A.B., 2007. An aryl hydrocarbon receptor odyssey to the shores of toxicology: the Deichmann Lecture, International Congress of Toxicology-XI. *Toxicol. Sci.* 98, 5–38.
- Pesonen, M., Goksoyr, A., Andersson, T., 1992. Expression of P4501A1 in a primary culture of rainbow trout hepatocytes exposed to  $\beta$ -naphthoflavone or 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Arch. Biochem. Biophys.* 292, 228–233.
- Peters, A.K., Leonards, P.E., Zhao, B., Bergman, A., Denison, M.S., van den Berg, M., 2006. Determination of *in vitro* relative potency (REP) values for mono-ortho polychlorinated biphenyls after purification with active charcoal. *Toxicol. Lett.* 165, 230–241.
- Powell, D.C., Aulerich, R.J., Meadows, J.C., Tillitt, D.E., Giesy, J.P., Stromborg, K.L., Bursian, S.J., 1996. Effects of 3,3',4,4',5-pentachlorobiphenyl (PCB 126) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) injected into the yolks of chicken (*Gallus domesticus*) eggs prior to incubation. *Arch. Environ. Contam. Toxicol.* 31, 404–409.
- Powell, D.C., Aulerich, R.J., Meadows, J.C., Tillitt, D.E., Kelly, M.E., Stromborg, K.L., Melancon, M.J., Fitzgerald, S.D., Bursian, S.J., 1998. Effects of 3,3',4,4',5-pentachlorobiphenyl and 2,3,7,8-tetrachlorodibenzo-p-dioxin injected into the yolks of double-crested cormorant (*Phalacrocorax auritus*) eggs prior to incubation. *Environ. Toxicol. Chem.* 17, 2035–2040.
- Regoli, F., Giuliani, M.E., Benedetti, M., Arukwe, A., 2011. Molecular and biochemical biomarkers in environmental monitoring: a comparison of biotransformation and antioxidant defense systems in multiple tissues. *Aquat. Toxicol.* 105, 56–66.
- Secord, A.L., McCarty, J.P., Echols, K.R., Meadows, J.C., Gale, R.W., Tillitt, D.E., 1999. Polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents in tree swallows from the upper Hudson River, New York State, USA. *Environ. Toxicol. Chem.* 18, 2519–2525.
- van den Berg, M., Birnbaum, L.S., Bosveld, A.T., Brunström, B., Cook, P., Feeley, M., Giesy, J.P., Hanberg, A., Hasegawa, R., Kennedy, S.W., Kubiak, T.J., Larsen, J.C., Van Leeuwen, F.X.R., Liem, A.K.D., Nolt, C., Peterson, R.E., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D.E., Tysklind, M., Younes, M., Wærn, F., Zacharewski, T.R., 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ. Health Perspect.* 106, 775–792.
- van den Berg, M., Birnbaum, L.S., Denison, M.S., DeVito, M.J., Farland, W.H., Feeley, M., Fiedler, H., Hakansson, H., Hanberg, A., Haws, L.C., Rose, M., Safe, S., Schrenk, D., Tohyama, C., Tritscher, A., Tuomisto, J., Tysklind, M., Walker, N.J., Peterson, R.E., 2006. The 2005 World Health Organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. *Toxicol. Sci.* 93, 223–241.
- Villeneuve, D.L., Blankenship, A.L., Giesy, J.P., 2000. Derivation and application of relative potency estimates based on *in vitro* bioassay results. *Environ. Toxicol. Chem.* 19, 2835–2843.
- Zhou, J.G., Henry, E.C., Palermo, C.M., Dertinger, S.D., Gasiewicz, T.A., 2003. Species-specific transcriptional activity of synthetic flavonoids in guinea pig and mouse cells as a result of differential activation of the aryl hydrocarbon receptor to interact with dioxin-responsive elements. *Mol. Pharmacol.* 63, 915–924.